AMINO ACID SEQUENCE DETERMINATION OF THE BLOCKED N-TERMINAL TRYPTIC PEPTIDE OF *NEUROSPORA* TYROSINASE BY MASS SPECTROMETRY

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1. Introduction

Tyrosinase (EC 1.10.3.1, *O*-diphenol : oxygen oxidoreductase) is a copper containing mixed-function oxidase catalyzing the *O*-hydroxylation of monophenols and the oxidation of *O*-diphenols. The enzyme has been isolated from a large variety of sources (for review see [1]) but very little is known on the structure of this oxidase, mainly due to its molecular heterogeneity. Tyrosinase from the ascomycete *Neurospora crassa* has recently been shown to consist of a single polypeptide chain mol. wt 44 000 thus opening the possibility of obtaining more information on its structure [2]. During sequence analysis of *Neurospora* tyrosinase in this laboratory it was found that the amino-terminal residue is blocked.

Since mass spectrometry has proved particularly useful for the identification of blocked peptides (cf. [3,4]), several small peptides derived from the amino-terminal cyanogen bromide fragment were analyzed by this technique. An acetyl blocking group was unambiguously identified and the sequence of the amino terminal tryptic pentapeptide was found to be *N*-acetyl–Ser–Thr–Asp–Ile–Lys.

In contrast to these results the amino terminal residues of all four isozymic forms of mushroom tyrosinase were reported to be isoleucine [5]. However, mushroom tyrosinase has been shown recently to be composed of two subunits [6] and therefore it is quite possible that one of the two subunits in this enzyme is also blocked.

2. Materials and methods

2.1. *Enzyme, isolation of peptides and analytical methods*

Tyrosinase from *Neurospora crassa* wild type strain (FGSC No. 320) was prepared according to [2]. The enzyme was cleaved with cyanogen bromide and the fragments separated on Sephadex G-100 and G-50 columns in 7% formic acid [7]. The blocked N-terminal cyanogen bromide peptide (1.0 μmol, 7.5 mg) was digested with TPCK-treated Trypsin (Worthington) at pH 8.6 and 37°C for 2 h by means of a Radiometer pH-stat. An enzyme/peptide molar ratio of 1:100 was chosen. The peptide mixture was fractionated on a 0.9 × 20 cm column of Beckman M-72 resin at 55°C using the pyridine–acetate buffer system [8]. The separation of peptides was monitored with a Technicon Autoanalyzer [9]. Peptides were further separated by chromatography on a 0.9 × 150

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cm column of Sephadex G-25 in 50% acetic acid and by high-voltage paper electrophoresis, at pH 1.9 [10]. Acid cleavage of the blocked peptides was performed in 0.25 M acetic acid in evacuated tubes for 18 h at 105°C. Carboxypeptidase B (Worthington) digestion of the blocked tryptic peptide was carried out at 37°C in 0.2 M *N*-ethyl-morpholine–acetate buffer, pH 8.5, for 1 h using an enzyme/peptide molar ratio of 1:100. Hydrazinolysis was performed according to [11] and the reaction mixture directly applied to the amino acid analyzer after lyophilyzation. Amino acid analyses of peptides were performed on a Durrum D-500 Analyzer after hydrolysis with 6 N HCl at 110°C for 20 h.

2.2. *Mass spectrometry and gas chromatography–mass spectrometry (GC–MS)*

The tryptic fragment (0.2 μmol) was methyl esterified by ethereal diazomethane and then permethylated by the procedure of Leclercq and Desiderio [12], but with a 3 min methylation time [13]. The permethylated sample was vaporized by the solid insertion probe directly into the MS-30 mass spectrometer (AEI), the temperature of the probe was raised manually up to a final value of 320°C while the mass spectrometer was scanned continuously (total scan time 7 s). The DS-50 data system (AEI) was used for the acquisition of mass spectra and for the calculation of mass chromatograms [14].

The peptide fragments generated by acetic acid hydrolysis of the blocked cyanogen bromide peptide and the blocked tryptic peptide were dissolved in 500 μl methanol containing two drops of water (samples of 0.4 μmol and 0.8 μmol, respectively, were used) and esterified by ethereal diazomethane. The methyl esterified peptides were reduced by 1 ml 1 N $LiAlD_4$ in dimethoxyethane at 85°C for 36 h [15]. The amino alcohols were isolated by the extraction procedure, then trimethylsilylated as described [16,17]. Portions of 5–10% of the derivatized mixtures were injected 'on column' into a Perkin-Elmer F-22 gas chromatograph; a 1 meter 63.5 mm o.d. and 2 mm i.d. glass column filled with 3% Se-30 on Suppelcoport 100/120 mesh was used and the temperature was raised from 60–340°C at a rate of 12.5°C/min. The column was coupled via a 1/4" Swagelock union and a membrane separator to the MS-computer system (see above). Three hydrocarbon standards were co-injected with the sample for the calculation of retention indices.

3. Results and discussion

In the course of sequence analysis of *Neurospora* tyrosinase, it became apparent that the denatured enzyme as well as the N-terminal cyanogen bromide fragment were both resistant to the automatic Edman degradation. These results were indicative of a blocked amino terminal residue in this enzyme. To determine the structure of the blocking group the amino-terminal cyanogen bromide fragment was cleaved with trypsin and the peptide mixture separated as outlined under 2.1. All peptides could be successfully subjected to sequence analysis (K. Lerch, in preparation) with the exception of an acidic pentapeptide. The amino acid composition of this peptide (T_1) is shown in table 1. 400 nmol of peptide (T_1) were treated with carboxypeptidase B and 0.95 residues of lysine/mol peptide were found after direct amino acid analysis.

A sample of 0.2 μmol of this mixture was permethylated and then vaporized into the mass spectrometer by slowly heating the direct insertion probe. The mass spectra obtained (not shown) were impure and difficult to interpret unambiguously. This is often the case if mixtures of permethylated peptides are analyzed directly by mass spectrometry since the mass spectra of a particular component may be obstructed by other peptide derivatives and particularly by everpresent impurities.

Therefore, mass chromatograms of selected ions were generated by the computer. First, all ions derived from N-acetylated and permethylated peptides containing either *N*-acetyl–Lys, –Asp, –Thr, –Ser, or

Table 1
Amino acid composition of tryptic and acid cleaved peptides of *Neurospora* tyrosinase

Amino Acid	T_1	T_1–H^+-1	H^+-1
Lysine	0.97 (1)	1.00 (1)	
Aspartic acid	1.00 (1)		
Threonine	0.97 (1)		1.00 (1)
Serine	1.06 (1)		0.91 (1)
Isoleucine	0.96 (1)	0.94 (1)	

Values are given in residues/mol. The assumed integral numbers are given in parentheses.

–Ile were plotted (fig.1). The ion indicating *N*-acetyl –Ser showed a conspicuous maximum around scan 100. Next, the penultimate amino acid residue was searched for by adding the masses [18] for Lys, Asp, Thr or Ile. Only ion m/e 255 indicating *N*-acetyl–Ser–Thr showed a maximum at scan 100. The dipeptide sequence thus obtained was extended analoguously to Ac–Ser–Thr–Asp (m/e 398) and then to Ac–Ser–Thr–Asp–Ile (m/e 526) (Ile rather than Leu was assigned using the amino acid analysis data of table 1).

By the interpretation of mass chromatograms in addition to mass spectra – to our knowledge this combined technique has hitherto not been used for the analysis of permethylated peptides – unambiguous peptide sequences can be obtained from peptide mixtures and in the presence of impurities.

Peptide T_1 was further treated with dilute acetic acid (see 2.1) and 0.9 mol. aspartic acid/mol peptide were found after direct amino acid analysis. The mixture was further separated by high-voltage electrophoresis (pH 1.9) and two major ninhydrin positive spots were obtained. One was identified as free aspartic acid, the other one was a dipeptide (T_1–H^+-1) with the amino acid composition as shown in table 1. A sample of 0.8 μmol of the above mixture was methyl esterified, reduced by $LiAlD_4$ and *O*-trimethylsilylated and the resulting mixture was analyzed by GC–MS. The gas chromatographic fraction eluting with a retention index of 1904 exhibited the mass spectrum shown in fig.2 which clearly indicates the sequence of *N*-Ac–Ser–Thr. The abundant ion m/e 162 indicates a peptide which *N*-acetyl–Ser at the amino-terminus (for the sequence ions and retention indices of *N*-acetylated and $LiAlD_4$-reduced peptides see [19]). Also the retention index measured for this GC-peak (1904) is in agreement with the index predicted for the derivative of *N*-acetyl–Ser–Thr on a SE-30 column. In addition the N-terminal cyanogen bromide fragment was cleaved with dilute acetic acid (see 2.1.) and subsequent separation of the peptide mixture on Sephadex G-25 yielded a ninhydrin negative dipeptide (H^+-1) with the amino acid composition as shown in the last column of table 1. The C-terminal residue of this dipeptide was found to be threonine by hydrazinolysis (0.5 residues threonine/mol peptide). This dipeptide (0.4 μmol) was analyzed by GC–MS as described above and a mass spectrum identical to that shown in fig.2 was obtained.

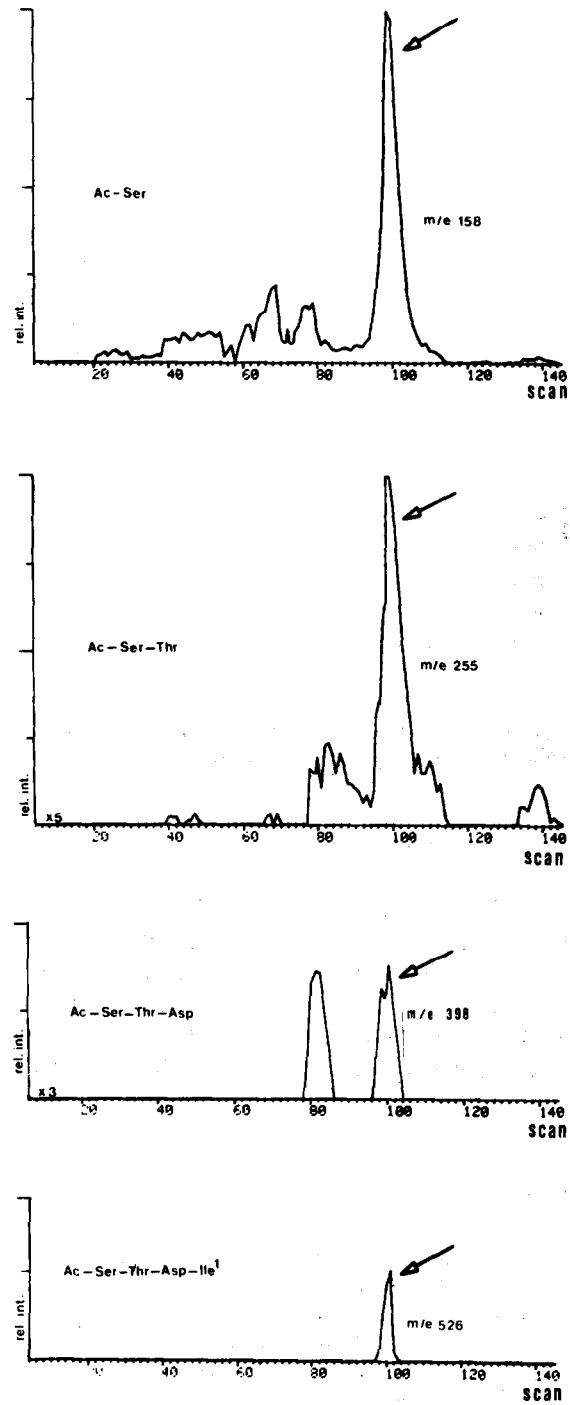

Fig.1. Mass chromatograms of sequence ions of the permethylated tryptic fragment. The sample was vaporized directly into the ion source of the mass spectrometer by manually heating the probe (scan 100 corresponds to 210°C).

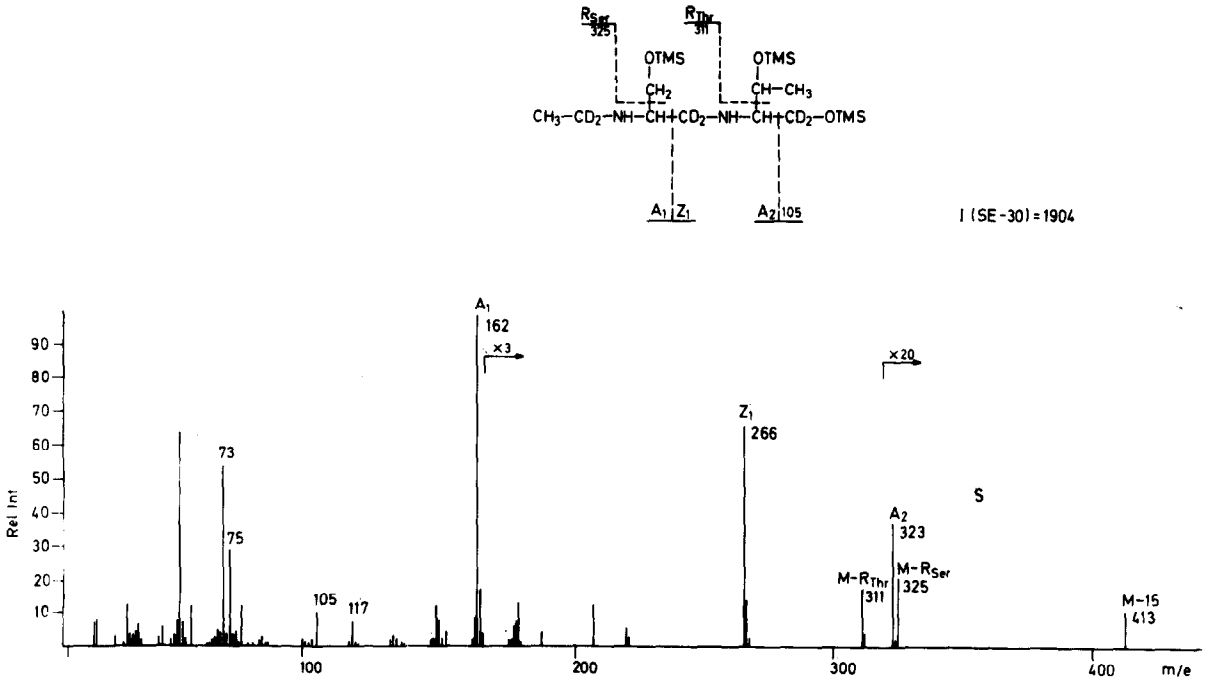

Fig.2. Mass spectrum of a gas chromatographic fraction obtained by methyl esterification, $LiAlD_4$-reduction and *O*-trimethylsilylation of the acetic acid hydrolysate of the tryptic fragment.

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